

Flow immunoassay for detection of wheat allergen using an anion exchange column and alkaline phosphatase conjugated IgE

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A simple and rapid detection system for wheat allergen was developed based on a luminescence immunoassay with a continuous flow system. Wheat allergen and alkaline phosphatase conjugated IgE (ALP-IgE) was separated from free ALP-IgE on the basis of a difference in isoelectric point, using with an anion exchange resin. Luminescence output of the assay correlated linearly with the concentration of allergen in the range of 1–100 µg/ml. Reuse of free ALP-IgE was possible.

Introduction

Adverse reactions to wheat flour pose a serious problem to human health. Recently the number of infants suffering from these disorders has increased. Many immunoassay methods have been developed for sensing or detecting allergens (Asero *et al.* 1996, Falagiani *et al.* 1994, Hoffman *et al.* 1996, Nakamura *et al.* 1995). However, there are presently neither simple nor rapid assay techniques for sensitive determination of wheat allergens in bulk-process systems.

Recently, we have developed a flow injection immunoassay which uses ion exchange column to separate labelled antibody-antigen complexes from free labelled antibody on the basis of a difference in isoelectric point (pI). This novel approach does not require prior immobilization of the antibody to a solid phase (Lim *et al.* 1995, 1997). The approach has several advantages of a much shorter assay time and a minimal sample volume. However the measurement has been restricted to only model allergen and the immunoassay system has not been automated.

In this paper, we have developed the flow immunoassay system equipped with an anion exchange column, luminescent reaction column and a photodetector. The IgE antibody with serum from a rat with a wheat allergy was purified by the use of DEAE-sepharose. In addition, ALP-IgE was employed for the detection of wheat allergen. To economize the system, reuse of ALP-IgE and the anion exchange column was also investigated.

Materials and methods

Materials

Sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (Sulfo-LC-SPDP) from Pierce (Rockford, IL). Alkaline phosphatase (ALP, from calf intestine) from TOYOBO (Osaka, Japan). Serum from a rat with a wheat allergy was obtained from Asahi Denka Kogyo K.K. (Tokyo, Japan). AMPPD was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals used were of analytical-reagent or laboratory grade. Deionized-distilled water was used in all procedures.

Purification of IgE from rat serum

10 ml of rat serum was dialyzed for 72 h at 4°C against 3 changes of 0.05 M Tris-HCl buffer (pH 8.0). Equal volumes of rat serum and Tris-HCl buffer were filtered through a DISMIC-25 cellulose nitrate filter (pore size 0.45 µm, ADVANTEC, Tokyo, Japan) then loaded onto a DEAE sepharose FF column (16 mm i.d. × 25 mm; Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min. Fractions were stepwise eluted with Tris-HCl buffer of increasing molarity (0.01, 0.02, 0.03, 0.05 and 0.1 M). Absorbance at 280 nm of the effluent was monitored. IgE and IgG in each fraction was detected by a flow injection luminescence immunoassay (Lim *et al.* 1995, 1997) using ALP conjugated IgG (anti-rat-IgE) and ALP conjugated IgG (anti-rat-IgG), respectively. In addition, NaCl was added stepwise (0.05, 0.1 M NaCl in 0.05 M Tris-HCl buffer) to increase the molarity of the buffer for elution beyond 0.05 M. The concentration of IgG and IgE in purified fractions was determined by the Bradford method

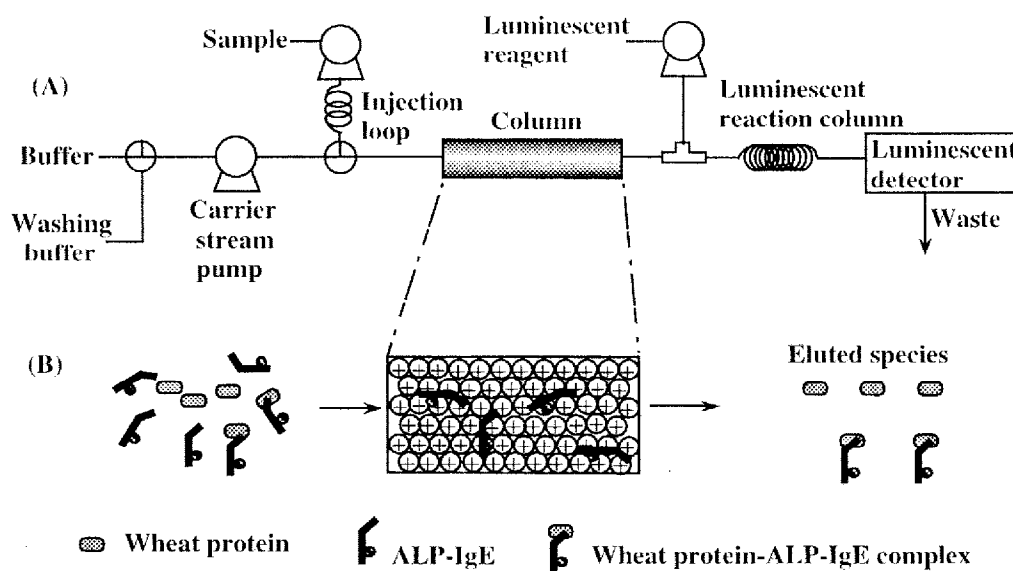


Figure 1 Schematic diagram of flow immunoassay system (A) and principle of flow immunoassay (B). Samples containing wheat allergen was loaded alongside ALP-IgE into a injection loop and incubated. The reaction mixture is passed through a column that selectively traps free ALP-IgE. The luminescence peak associated with the wheat allergen-ALP-IgE complex is monitored.

(Bradford 1976, Compton and Jones 1985) (Bio-Rad Laboratories, Hercules, CA).

Preparation of ALP conjugated IgE antibody, wheat flour extracts and allergen-reduced wheat

ALP conjugated IgE antibody (ALP-IgE) was prepared by reacting reductively-cleaved IgE with pyridyl disulfide derivatized alkaline phosphatase. Purified anti wheat IgE (100 μ l, 0.5 mg/ml) was reductively cleaved by incubation for 2 h at room temperature with 20 mM dithiothreitol (100 μ l) dissolved in phosphate buffer (containing 5 mM EDTA, pH 7.0). Reductively-cleaved IgE was desalted on a HiTrapTM Column (16 mm i.d. $\times$ 25 mm; Pharmacia), using phosphate buffer (pH 7.0) according to the manufacturer's instructions. Pyridyl disulfide groups were introduced into alkaline phosphatase using Sulfo-LC-SPDP (Carlsson *et al.* 1978, Peeter *et al.* 1981). Sulfo-LC-SPDP (0.5 mg) was added to 1 ml of ALP solution (0.6 mg/ml in phosphate buffer containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) and incubated for 2 h at 24°C. The concentration of IgE was determined by the Bradford method before and after conjugation. The ALP conjugated anti wheat IgE prepared in this manner was homogeneous as determined by gel filtration on Sephacryl S-300. ALP-IgE was stable at 4°C for several weeks.

Commercially available wheat flour was extracted by a modification of the method of Simmonds and Wrigley

(1972). Allergen-reduced wheat was prepared by a modified method of previous study (Nakamura *et al.* 1995).

Apparatus

A schematic diagram of the flow luminescence immunoassay apparatus is shown in Fig. 1. It consists of the anion exchange column, the luminescent reaction column, the luminescent detector, a carrier stream pump (Model 011-01, FLOM, Tokyo, Japan), two peristaltic pumps (U4-XV, ALITEA, Stockholm, Sweden), a three-port clean valve (EXAK-3, TAKASAGO, Nagoya, Japan) and a six-port injection valve (US09/V49, FLOM, Tokyo, Japan) fitted with a 100 μ l loop. The luminescent reaction column (stainless steel coil; 1 mm i.d. $\times$ 16 m) was used to incubate luminescent reagent and wheat protein-ALP-IgE. The luminescent reaction columns were maintained at 37°C in a thermostatic incubator. The luminescent detector was interfaced to photosensor amplifier (C2719, Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). All tubing except the stainless steel coil was 0.5 mm i.d. TEFZEL tube from Pharmacia. Luminescence intensities were calculated by an IBM computer using the MPCA-30s software package from DKK Corporation (Tokyo, Japan).

Continuous flow immunoassay using anion exchange column

Samples containing wheat allergen (50 μ l) was loaded alongside 50 μ l of 7 μ g/ml ALP-IgE into a 100 μ l injection loop, and incubated 15 min. The immuno-

reaction products were passed through an anion exchange column packed with Sepharose Fast Flow (quaternary ammonium type, 16 mm i.d. $\times$ 25 mm; Pharmacia) in a stream of 20 mM *N*-methylpiperazine buffer (pH 4.3) at a flow rate 1.0 ml/min. After passage of the first sample loaded onto the anion exchange column, the next sample was loaded into the injection loop. The combined effluent from the anion exchange column and luminescent reagent was passed through the luminescent reaction column at an overall flow rate of 1.0 ml/min for the purposes of incubation then into a luminescent detector.

Reuse of free ALP-IgE bound to the anion exchange resin

The anion exchange column was regenerated by elution with 20 mM *N*-methylpiperazine buffer (pH 4.3) containing 0.5 M NaCl to remove bound free ALP-IgE conjugate was passed through a desalting column to remove NaCl. A sample of this recycled ALP-IgE (50 μ l, 7 μ g/ml) was assayed for immuno-reactivity by flow immunoassay against a fixed quantity of wheat protein (50 μ l, 100 μ g/ml). This procedure was repeated with successive eluted free ALP-IgE and the relationship between regeneration cycle and luminescence signal ascertained.

Results and discussion

Purification of IgE

Rat serum with elevated IgE was used to optimize the IgE purification method. After extensive dialysis, a sample of rat serum was applied to a DEAE-cellulose column. Fractions eluted by stepwise increase in molarity of the elution buffer, were analyzed for protein by measurement of absorbance at 280 nm, and for IgE and IgG by immunoassay. The major protein and IgG peaks were detected in the unbound fraction Fig. 2A (fraction 1–4). IgE was eluted at an ionic strength of 0.1 M. Smaller stepwise increases in the salt concentration were used to improve the separation of IgE from other serum proteins. Non-specific proteins and IgG (90 % of the total IgG) were eluted in a broad peak as shown in Fig. 2B (fraction 1–6). By contrast, IgE was eluted as a sharp peak with 0.05 M Tris-HCl buffer containing 0.05 M NaCl. Further increases in ionic strength eluted a broad peak of non IgE proteins, together with trace quantities of IgE and IgG (Fig. 2B). Based on these results, a good separation of IgE from other serum proteins was performed using 0.05 M Tris-HCl buffer containing 0.05 M NaCl (pH 8.0) on a DEAE sepharose FF column.

Preparation of ALP-IgE conjugate

The number of pyridyl disulfide groups incorporated per molecule of ALP was dependent on the amount of linker used during derivatization (Husain and Bieniarz 1994).

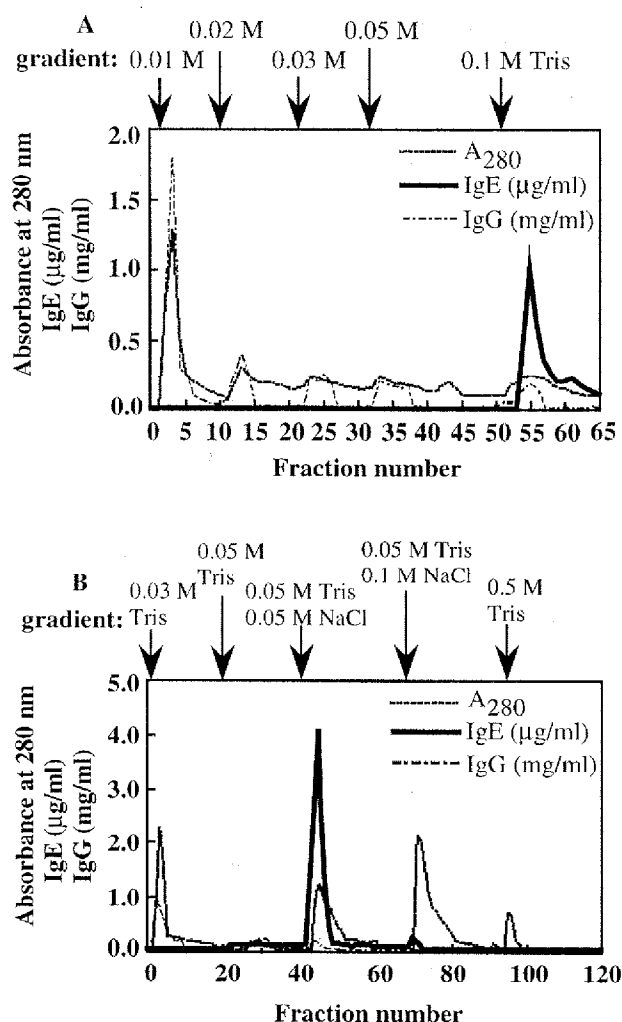


Figure 2 Chromatograms are presented based on two different methods for anion exchange purification of IgE from serum using DEAE sepharose FF column. Each fraction contains 1 ml of eluent. (A) molarity of Tris (pH 8.0) was stepwise increased to separate IgG and other serum proteins from the IgE fraction. (B) to enhance the separation of IgE from other serum proteins, NaCl was added in a stepwise manner to the Tris-HCl buffer.

The number as determined spectrophotometrically using the adsorption coefficient of mercaptopyridine and ALP, and the molecular weight of ALP (100,000) (Engström 1961). The result showed the incorporation of four pyridyl disulfide groups per ALP molecule. Enzyme activity of derivatised ALP was 87 % of native ALP. This is consistent with Husain and Bieniarz (1994) who reported a 20 % decrease in enzymic activity after incorporation of 13 pyridyl disulfide groups per molecule. Derivatized ALP with four pyridyl disulfide groups per enzyme molecule was routinely used to prepare ALP-IgE conjugate.

Separation of ALP-IgE and wheat allergen-ALP-IgE complex by anion exchange column

A method was required to separate ALP-IgE conjugate from wheat allergen-ALP-IgE complex. Isoelectric gel electrophoresis was correspondingly used to determine any useful difference in isoelectric point of these species. Wheat allergen, ALP-IgE conjugated and wheat allergen-ALP-IgE complex were analyzed by isoelectric gel electrophoresis using the PhastGel™ IEF 3–9 system and found to have pI values of 6.0 to 7.35, 4.0 and 6.0 respectively. A significant difference in pI of ALP-IgE (4.0) and wheat allergen-ALP-IgE (6.0) was noted. We therefore attempted to separate these two species by anion exchange on Sepharose Fast Flow (quaternary ammonium type) equilibrated at pH 4.3 with 20 mM *N*-methylpiperazine buffer. Free ALP-IgE was applied to the anion exchange column and eluted with 20 mM *N*-methylpiperazine buffer (pH 4.3). No peak was observed. Therefore, free ALP-IgE was retained by the anion exchange resin. ALP-IgE (50 μ l, 7 μ g/ml) and wheat allergen (50 μ l, various concentration) were simultaneously loaded to the luminescence flow immunoassay system. This procedure was repeated intervals of 15 min. After a lag of 15 min, a series of single peaks spaced 15 min apart were obtained from the luminescent detector (Fig. 3).

Wheat allergen-ALP-IgE complex was not retained by the anion exchange column. The signal from the flow immunoassay increased with increasing concentration of wheat

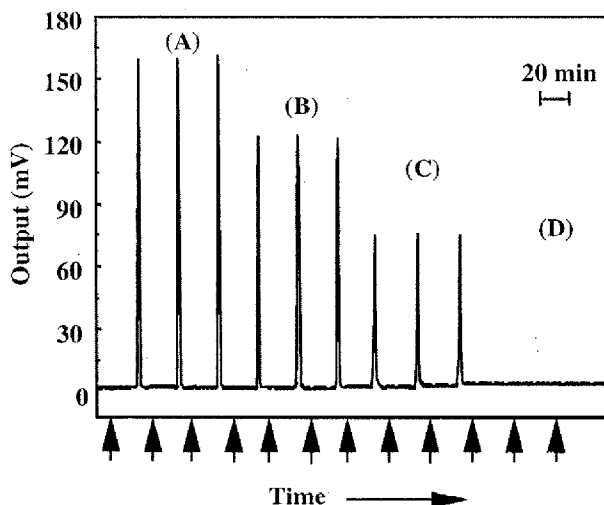


Figure 3 Continual flow injection analysis. (A) 100, (B) 70, (C) 40 and (D) 0 μ g/ml wheat protein were reacted with 7 μ g/ml ALP-IgE, and loaded onto the system. Arrows indicated the sample injection. Eluent; 20 mM *N*-methylpiperazine buffer (pH 4.3). Flow rate; 1 ml/min.

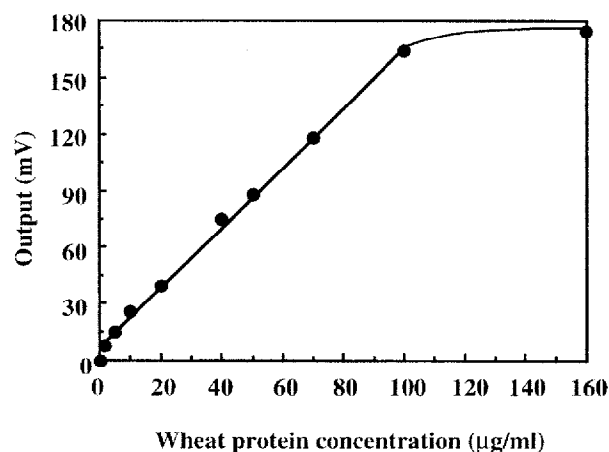


Figure 4 Calibration for wheat allergen concentration. ALP-IgE; 7 mg/ml. $R = 0.997$ (1–100 μ g/ml)

allergen. AMPPD did not yield a significant background as revealed by the slight baseline elevation in all analyses.

Calibration for wheat allergen concentration

A typical calibration curve using wheat allergen is shown in Figure 4. Signal output (mV) by the luminescent detector increased with increasing concentration of wheat allergen. A linear relationship was obtained between signal output and concentration of wheat allergen in the range of 1 to 100 mg/ml. The correlation coefficient was 0.997 within this range. This method is faster (15 min) and simpler to use than other immunoassays, and gives greater precision (less than 2 %) as compared to a conventional ELISA using microtiter plates. Reproducibility of the immunoassay was investigated using five different concentration of wheat allergen (5, 20, 40, 55 and 100 μ g/ml). The coefficients of variation for each concentration were all in the range 1.3% to 2.2%. Data from this analysis showed good correlation ($R = 0.995$, $n = 10$). The immunoassay was reproducible ($n = 10$) and sensitive.

Regeneration of anion exchange column and reuse of free ALP-IgE bound to the anion exchange resin

The anion exchange column was regenerated by elution with *N*-methylpiperazine buffer (pH 4.3) containing 0.5 M NaCl after 15 times continuous monitoring of allergen-ALP-IgE complex. This resulted in the appearance of one peak after 5 min. We investigated the relationship between the extent to which ALP-IgE had been recovered from the anion exchange column by elution with *N*-methylpiperazine buffer and subsequently desalted, and responsivity of the immunoassay when assaying a fixed quantity of wheat protein allergen using recycled ALP-IgE.

Table 1 Specificity of the measurement using ALP conjugated IgE

Protein	Output (mV)
None	0.0
Wheat	155.0
Allergen reduced wheat	23.0
BSA	1.1
IgG	1.2

ALP conjugated IgG; 7 µg/ml
Concentration of protein; 100 µg/ml

ALP-IgE may be reused up to five times without significant effect to efficiency of the immunoassay.

Specificity for various proteins

The specificity of the flow immunoassay for wheat protein allergen was investigated by assaying a variety of other proteins (Table 1). The immunoassay only yielded significant signal when using wheat allergen.

Conclusion

We present a novel flow immunoassay for detection of wheat allergens. An IgE antibody is isolated from serum of a rat with a wheat allergy, reductively-cleaved using dithiothreitol then conjugated to alkaline phosphatase. Unreacted ALP-IgE is separated from allergen-ALP-IgE complex on the basis of a difference in pI by ion exchange column. This system allowed optimization of reaction conditions, including antibody concentration compared

with ordinary solid phase immunoassay system, assay time is reduced. Reuse of free ALP-IgE is also possible with this system. The data show that this is accurate and reproducible measurement.

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